Rosacea, Reactive Oxygen Species, and Azelaic Acid

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ABSTRACT

Rosacea is a common skin condition thought to be primarily an inflammatory disorder. Neutrophils, in particular, have been implicated in the inflammation associated with rosacea and mediate many of their effects through the release of reactive oxygen species. Recently, the role of reactive oxygen species in the pathophysiology of rosacea has been recognized. Many effective agents for rosacea, including topical azelaic acid and topical metronidazole, have anti-inflammatory properties. *In-vitro* models have demonstrated the potent antioxidant effects of azelaic acid, providing a potential mechanistic explanation for its efficacy in the treatment of rosacea. (*J Clin Aesthetic Derm.* 2009;2(1):26–30.)

Rosacea is a common skin condition with both genetic and environmental factors involved in its etiology and a pathophysiology that may involve inflammatory mediators and reactive oxygen species (ROS).[1–9] The hallmark of rosacea is central facial erythema.[1,3] Patients with this vascular sign are categorized as having subtype 1, or erythematotelangiectatic rosacea, involving persistent erythema and telangiectases (dilated superficial veins). Some patients may progress to subtype 2 rosacea, or papulopustular rosacea, characterized by the development of acne-like papules and pustules. In subtype 3 rosacea, or phymatous rosacea, facial tissue hyperplasia is also evident. Rosacea does not necessarily progress from one stage to another or to a more severe form.[1,3,4] It has a varying natural history. However, all stages of rosacea have two consistent characteristics: tendency for facial flushing and sun damage, especially solar elastosis.[1]

In 2002, the National Rosacea Society committee identified four major subtypes of rosacea: the three subtypes previously mentioned in addition to subtype 4, or ocular rosacea.[3,4] This was seen as a first step toward standardizing rosacea therapy.[4,10]

Rosacea has previously been considered primarily a cutaneous vascular disorder.[11] However, with the rapidly growing understanding of immune system processes in the skin and the success of anti-inflammatory agents in the treatment of rosacea, the role of inflammatory cells and mediators as key pathophysiologic factors in the development of rosacea has been recognized.[1,2,5] Utilizing inflammation as a central organizing principle, researchers are now commonly focusing on links between separate components, such as the recent demonstration of vascular endothelial growth factor (VEGF) as a link between ultraviolet light B (UVB) damage, vasodilation, and inflammation in the skin.[12]

POTENTIAL INFLAMMATORY MECHANISMS OF REACTIVE OXYGEN SPECIES IN ROSACEA

Neutrophils, in particular, have been implicated in the inflammation associated with rosacea.[1,2,5–8] Intrafollicular neutrophils are prominent in papulopustular rosacea, and it has been postulated that neutrophil-mediated inflammation underlies many of the varying manifestations of rosacea. Proteases released by neutrophils may degrade elastin and collagen, causing a compromised lymphatic system in the skin and compromised integrity of capillary walls.[2,8] If protein exudes from compromised capillaries and cannot be cleared from the interstitial area by a compromised lymphatic system, it could contribute to fibroplasia and ultimately to the rhinophyma seen in severe rosacea.[2]

Neutrophils are an important source of ROS early in the inflammatory process. ROS play a role in the damage seen with photoaging, an etiologic factor in the development of rosacea.[1,2,5,7,13] ROS also induce VEGF directly.[14]

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ROS include free radicals (atoms or molecules with an unpaired electron) and other reactive molecules, such as molecular oxygen, singlet oxygen, and hydrogen peroxide (H_2O_2), that are capable of initiating oxidative reactions and generating free radicals.[13,15] Free radicals, such as superoxide anions and hydroxyl radicals, are extremely chemically reactive, short-lived, and tend to cause the most oxidative tissue damage.[15]

Several biochemical mechanisms may be involved in the inflammation that characterizes rosacea. One is the deactivation of natural defenses by overwhelming oxidant stress from ROS.[13,15] Defense against oxidative damage by UV-generated free radicals is mediated by several pathways, including the thioprotein/thioredoxin reductase system, in both guinea pig and human skin.[16] The thioredoxin reductase/thioredoxin system reduces superoxide radicals through H_2O_2 to water.[16] However, Sundaram et al[16] have reported that both UVA and UVB radiation even below the minimum erythema dose generate a high-enough concentration of oxygen radicals to deactivate this enzyme-defense system considerably: Thioredoxin reductase was deactivated by 54 percent with UVA and by 34 percent with UVB radiation.[16] The release of superoxide dismutase (SOD), an enzyme with antioxidant activity, is another mechanism providing defense against oxidative damage. In a study of levels of SOD and malondialdehyde (MDA, a marker of oxidative tissue damage) in rosacea, severe rosacea was associated with lower SOD levels and higher MDA levels, suggesting that in severe rosacea, oxidative defense by SOD can be overwhelmed.[17]

Another possible trigger of skin inflammation is the oxidative modification of proteins and lipids by ROS. Chemical modification of proteins with products of lipid peroxidation can generate aldehyde-derived protein adducts (for example, MDA-lysine). Similarly, esters detected during the lipoxidation of linoleic acid can form reactive protein-lipid adducts. Although their role in the inflammation of rosacea is unknown, data suggest that lipoxidative modification of proteins occurs endogenously and is associated with the pathogenesis of atherosclerosis and other conditions associated with oxidative stress through uptake by scavenger receptors on macrophages.[18,19]

A third possible mechanism of skin inflammation in rosacea is an altered lipid balance in affected individuals. Linoleic acid is an essential skin component that markedly suppresses neutrophil ROS generation and phagocytosis.[20] One group of investigators examined the effect of palmitic acid on inflammatory parameters, such as neutrophil chemotaxis, phagocytosis, and ROS generation.[20] In this study, a cell-free xanthine-xanthine oxidase system was used to evaluate palmitic acid effects on ROS, including superoxide radical anions (O_2-), H_2O_2, and hydroxyl radical (OH). Palmitic acid was found to significantly decrease H_2O_2 generation by neutrophils in the xanthine-xanthine oxidase system, while neutrophil chemotaxis and phagocytosis, as well as the generation of both O_2- and OH by both systems, were not markedly affected.[20] The study implicates palmitic acid in oxidative tissue injury and suggests that palmitic acid may be involved in the pathogenesis of acne.[20] Further study of the proportions of linoleic and palmitic acid in the skin of rosacea patients may be warranted.

A fourth possible mechanism through which ROS can contribute to the inflammation associated with rosacea is through the antimicrobial peptide cathelicidin LL-37, which has recently been linked to the production of ROS.[21] Zheng et al[21] examined ROS generation (via flow cytometry) and found that LL-37 stimulated the generation of ROS both dose and time dependently, likely through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and intracellular Ca_2+ mobilization.[21] This suggests that in addition to its antimicrobial properties, LL-37 also may contribute to innate immunity via enhancement of neutrophil host defense functions at specific inflammation and/or infection sites.[21] Further, antimicrobial peptides, such as cathelicidins and defensins, may act in a dual fashion by acting both to kill microbes and to trigger host tissue responses.[22] Since many of the effects of cathelicidin are similar to clinical changes seen in rosacea (e.g., abnormal inflammation, vascular reactivity), Yamasaki et al[22] hypothesized that the abnormal expression of cathelicidin peptides may be implicated in the pathogenesis of rosacea.[22] In testing this hypothesis, it was found that patients with rosacea express abnormally high levels of cathelicidin (LL-37 peptide form) in their facial skin; whereas, patients with normal facial skin showed minimal expression.[22] Moreover, cathelicidin was diffusely found throughout the epidermis of individuals with rosacea, but not so in healthy volunteers.[22] The central role of cathelicidin was further supported in a subsequent study of mice.[22]

A fifth mechanism by which ROS can contribute to inflammation in rosacea is through cytokines and other inflammatory mediators. ROS-mediated damage to keratinocytes, fibroblasts, and endothelial cells can result in the release of mediators such as IL-1 and TNF-α, which are potent modulators in the recruitment of leukocytes from the blood to the tissues.[23]

GENETIC AND AGE-RELATED FACTORS SUPPORT A ROLE FOR ROS

Although genetic effects on rosacea development remain largely uncharacterized at the molecular level, a recent study examined the relationship between specific genotypes and the development of rosacea.[24] The investigators hypothesized that increased ROS activity or decreased antioxidant potential, possibly induced by a glutathione S-transferase (GST) gene polymorphism, might have a role in the pathogenesis of rosacea. GST plays a primary role in cellular defense against electrophilic chemical species and ROS.[24] When DNA samples from 45 rosacea patients were compared with 100 control subjects using the polymerase chain reaction (PCR), null mutations

in polymorphic glutathione S-transferases GSTM1 and GSTT1 genotypes were significantly associated with rosacea development.[24] While intriguing, further studies in larger patient groups must be done before suggesting the use of GST mutations as markers of rosacea susceptibility. However, these results add greater weight to the idea of ROS as an underlying cause of rosacea and suggest that an underlying deficiency in normal antioxidant defense mechanisms may be a contributing factor to rosacea pathophysiology.[24] A recent study documented an age-related decrease in endogenous ascorbic acid (vitamin C) in skin.[25] Vitamin C is known for its antioxidant potential and activity in the collagen biosynthetic pathway.[15,25] Lower levels of this antioxidant in older skin coupled with the observation that rosacea is a condition that develops with age also support the idea that ROS play key roles in the pathogenesis of rosacea.[15,25]

IN-VITRO METHODS FOR MEASURING ROS SUGGEST DIFFERENT OXIDATIVE PATHWAYS

There are a number of *in-vitro* methods to evaluate the effects of different dermatological agents on ROS. Different tests can yield different results, depending on the particular oxidative pathways involved.[26,27] One standard evaluation method uses a preparation of neutrophils stimulated with zymosan to produce an oxidative burst.[13,28] Neutrophilic ROS generation is determined by evaluating changes in the light absorbance, fluorescence, or chemiluminescence of various added markers caused by O_2-, H_2O_2, or OH, respectively.[28] Adding various concentrations of an agent to this cellular system and comparing with known antioxidant controls determines whether an added agent can inhibit the production of ROS by neutrophils.[28] This assay system is often paired with the xanthine-xanthine oxidase system, a chemical reaction used to generate ROS in a cell-free environment, in order to detect the scavenging ability of a compound.[13,28] If a compound is found to inhibit ROS development in the cellular system, but not the cell-free system, it is thought that the effect of the compound is to inhibit the cells' production of ROS. Conversely, if the compound has an effect in the cell-free system, it is thought to exhibit scavenging effects rather than inhibitory effects on cell-mediated ROS generation.[13,28] This is a somewhat simplified explanation, as there are various different ROS-related processes and many different scavenging/quenching effects of antioxidants.

MANY EFFECTIVE AGENTS FOR ROSACEA HAVE ANTI-INFLAMMATORY PROPERTIES

Many of the effective agents for rosacea have evidence of anti-inflammatory effects. In the case of metronidazole, studies have examined antioxidant and other anti-inflammatory properties of metronidazole.[2,29] In the neutrophil ROS system, H_2O_2 inhibition by metronidazole was dose dependent, with borderline significance achieved at 10µg/mL and true significance at 100µg/mL.[2] The production of OH- was significantly suppressed at both 10 and 100µg/mL.[2] However, metronidazole had no effect on ROS generated in the cell-free system, suggesting that metronidazole exerts its effects by modulating neutrophils rather than direct oxidant scavenging.[2]

A subsequent *in-vitro* trial combined various combinations of metronidazole with palmitoleic acid, a free fatty acid found in skin.[2,29] Metronidazole alone slightly decreased H_2O_2 and OH-, but when metronidazole was combined with palmitoleic acid, O_2- and other neutrophil-generated ROS were significantly suppressed in a dose-dependent manner.[2,29] ROS generation in the cell-free system was unaffected, and there was no effect on neutrophil chemotaxis or phagocytosis.[2,29]

Oral antibiotics are used more often for severe forms of inflammatory rosacea and as induction therapy with topical agents such as metronidazole.[30,31] Tetracycline antibiotics are a mainstay of both acne and rosacea therapy and have anti-inflammatory as well as antimicrobial properties.[28,31] Tetracyclines effectively inhibit the generation of H_2O_2 and other oxygen radicals by neutrophils.[28] The anti-inflammatory effects of tetracycline antibiotics have been validated more recently with the use of subantimicrobial doses of doxycycline and other tetracycline antibiotics in acne and rosacea.[32] Based on subjective and objective findings, clinical efficacy with 20-mg bid doxycycline was very similar to efficacy seen with standard antibiotic dosages.[32]

Both oral and topical retinoids have also been shown to have anti-inflammatory effects.[33] Low-dose isotretinoin and topical retinoids have demonstrated benefits in rosacea treatment, although clinical response may not be evident until two or more months into treatment.[33,34]

Results from a study of a novel oral formulation of the antioxidant nicotinamide 750mg and zinc 25mg in patients with acne or rosacea showed that a significantly greater number of patients reported improvement in inflammatory lesions after a relatively short four weeks of treatment than those who reported worsening or no change.[36] Seventy-nine percent of the patients studied reported their improvement in appearance as "moderately better" or "much better," as measured by patient global evaluation, and 55 percent reported moderate or substantial improvement after four weeks of treatment ($P<0.0001$).[35]

AZELAIC ACID INHIBITS PRODUCTION OF ROS AND PROTECTS FROM ROS-MEDIATED DAMAGE *IN VITRO*

Azelaic acid (AzA), a naturally occurring dicarboxylic acid, is the newest topical addition to the rosacea armamentarium. This agent has been available for some time in Europe as a 20% cream, and has been more recently formulated as a 15% hydrogel. AzA reduces inflammatory lesions and erythema in rosacea patients and also inhibits neutrophilic ROS similarly to metronidazole.[31,36]

In the neutrophil system, AzA inhibits generation of ROS in a dose-dependent manner, markedly decreasing O_2- and OH, and decreasing H_2O_2 to a lesser extent (Table 1).[33] In the xanthine-xanthine oxidase system, none of the ROS generated was decreased by any dose of AzA, indicating that AzA does not scavenge generated ROS, but rather inhibits cell metabolism, possibly by decreasing enzymatic activity within the cell membrane. This study found that NADPH oxidase activity on the neutrophil surface membrane, which mediates the neutrophilic production of most ROS, is effectively inhibited by lower concentrations of AzA.[36]

AzA has been shown to affect a number of enzymes, including tyrosinase, the key enzyme of melanogenesis, and is capable of significantly inhibiting the hydroxylation of aromatic compounds and the peroxidation of arachidonic acid due to reactive hydroxyl ions.[26,37] This ability of AzA is quantifiable only in conditions of high OH concentrations, and is especially evident in reactions catalyzed by UV radiation.[26,36,37]

TABLE 1. Mean effects of azelaic acid on ROS in a neutrophil-zymosan system

AZELAIC ACID CONCENTRATION, µg/mL	O_2 LEVELS (nmol/min/1x10^6 NEUTROPHILS)	H_2O_2 LEVELS (pmol/min/2.5x10^5 NEUTROPHILS)	OH-LEVELS (pmol/min/3x10^5 NEUTROPHILS)
0	2.90	1.90	1.65
0.05	2.75	0.99*	1.55
0.5	2.60	1.00*	1.30†
5.0	2.30†	1.15*	1.20†
50.0	2.20‡	1.10*	0.50§

*P<0.05, † P<0.01, ‡P<0.001, §P<0.0001 versus control

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The ability of AzA to protect human cell lines from oxyradical-induced toxicity was assessed by either adding polyphenols directly to the cell growth medium to generate oxyradicals or by irradiating the phosphate-buffered saline in which the cells were incubated for 15 minutes prior to incubation in normal medium.[27] AzA significantly decreased the polyphenol toxicity toward cell lines for up to 24 hours.[27]

In the UV-irradiated cell lines, AzA was capable of protecting cells against UV damage, even after 48 hours of incubation.[27] UV cytotoxicity depends mainly on the generation of HO which can be scavenged by AzA.[27,37]

CONCLUSION

Although the pathophysiology of rosacea is multifactorial and incompletely understood, there is increasing consensus that inflammation is a central process in rosacea. This inflammation is linked through a variety of mechanisms to other processes including UV damage, vascular changes, and oxidative tissue damage. The most effective agents against rosacea have anti-inflammatory properties. As with topical metronidazole, AzA demonstrates significant anti-inflammatory activity in established *in-vitro* assays, and is able to inhibit neutrophil metabolic function and the release of ROS. This mechanism of action could explain the efficacy of AzA and topical metronidazole seen in clinical trials in rosacea[38–40] and suggests avenues for further research.

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